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Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss Dairy Cattle by Culture and Serology

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Inhaltsverzeichnis

1. Manuskript

Summary

Fecal samples from 186 dairy cows representing ten commercial dairy herds with sporadic clinical paratuberculosis (group A), and from 100 dairy cows from herds without a history of paratuberculosis (group B) were cultured for *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Two different decontamination methods, a NaOH/oxalic acid method and treatment with 0.75% hexadecylpyridinium chloride (HPC) were performed prior to inoculation of Loewenstein-Jensen agar slants with and without mycobactin. Cultures were incubated for 16 weeks. Acid-fast staining bacteria (AFB) were identified as MAP on the basis of mycobactin dependency and by PCR-coupled RFLP analysis of the IS*1311*-insertion element of *M. avium*. MAP was grown from 15 of 186 group A animals (8.1%) whereas fecal culture for MAP was consistently negative in group B. In comparison with HPC-treated fecal samples, the growth rate of MAP was significantly higher (8.1% vs. 1.6%) and the contamination rate of cultures was significantly lower (17.6% vs. 21.5%) in fecal samples decontaminated with NaOH/oxalic acid ($p<0.01$, McNemar's test). Atypical mycobacteria which were grown from 46.8% of NaOH/oxalic acid treated specimens were not obtained from any of the HPC-treated samples. A commercial ELISA with MAP-lipoarabinomannan (LAM) as antigen was used to detect MAP-antibodies in unabsorbed sera from all animals. The percentage of ELISA-positive cows was 16.8%. The overall agreement between antibody detection and MAP-positive fecal culture was only poor with 15.4%. The validity of positive ELISA reactivities in MAP-culture negative animals is suggested since ELISA values of animals with both, a positive AFB microscopy and growth of atypical mycobacteria (n=31), were consistently negative and did not differ significantly from corresponding values of animals with negative AFB microscopy as well as negative mycobacterial culture (n=26; $p=0.157$, Mann-Whitney test).

Introduction

Mycobacterium avium subspecies (ssp.) *paratuberculosis* (MAP) is the causative agent of paratuberculosis (Johne's disease), a granulomatous incurable enteropathy affecting primarily wild and domestic ruminants. The disease has been established as an increasingly important cause of morbidity and significant economic loss in the dairy and beef cattle industry (Buergelt and Duncan, 1978; Jones, 1989; Merkal et al., 1975; Nordlund et al., 1996). In Switzerland, data about the clinical and economic impact of bovine paratuberculosis are scarce. The disease is diagnosed only rarely so far with on average six outbreaks per year registered by the Swiss Federal Veterinary Office (FVO) (http://www.bvet.ch/admin). Nearly nine years ago, a herd-level seroprevalence of 8.0% was determined in a serosurvey at nationwide level (Stärk et al., 1997). The very recently reported PCR amplification of the MAP-specific IS*900* gene from 19.7% of 1384 bulk milk samples from different regions in Switzerland provided presumptive evidence for an increase of the prevalence of subclinical MAP-infections in the Swiss dairy stock (Corti and Stephan, 2002). Bovine paratuberculosis tends to cluster by age in groups of animals because exposure to MAP follows temporal patterns as multiple calves are exposed to infectious feces or milk from a shedding cow (Kalis et al., 2000), with clinical disease following only years after initial infection. Thus, data indicating an increasing prevalence of subclinical MAP-infections should receive our attention because of the possible risk of a future increase of clinical paratuberculosis in dairy stocks. Animals in the end stage of the disease, which is characterised by wasting due to chronic diarrhea, are shedding high levels of MAP in feces, thereby providing increased opportunities for transmission to other animals.

Various assays for the diagnosis of MAP-infections in cattle have been established, including Ziehl-Neelsen staining of fecal smears, histopathologic examinations, detection of MAP-antibodies in blood serum and milk, intradermal Johnin test or, alternatively, in vitro assays for cytokines, PCR-amplification, and culturing, with culture of MAP from affected tissues and feces being the “goldstandard” (Chiodini et al., 1984). Control of bovine paratuberculosis is achieved by identification and culling of infected hosts, with diagnosis mainly being based on serology and culture (Collins, 1996). The sensitivity of serology and culture may seem low, because animals passing through the long preclinical stage of infection generally have undetectable levels of MAP-specific antibodies and shed

undetectable to low numbers of MAP in feces (Collins, 1996; Stabel, 1997). Nonetheless, those animals are an important source of infection. Thus, diagnosis of subclinical paratuberculosis presents a major problem in the control of bovine paratuberculosis. MAP-infected cattle may also have public health implications since the agent has been linked to a human chronic granulomatous ileitis (Crohn's disease). However, the pathogenic role of MAP in humans remains controversial up to now (Hermon-Taylor, 2001; Quirke, 2001; Grimes, 2003).

Considering the pathogenic significance in cattle as well as the public health implications due to the probable involvement of MAP in Crohn's disease, current efforts are made in Switzerland to analyse the prevalence of MAP in the cattle population as an assistance in reaching a decision whether a control program needs to be established. The present study was aimed to adopt diagnostic protocols which are in use in other European countries to detect MAP-infections in cattle, and our report outlines a comparison of two methods for culturing MAP from bovine fecal samples and the application of an ELISA to examine bovine blood serum for MAP-antibodies.

Materials and methods

Animals and samples

Two groups of animals were enrolled in the study. Group A consisted of 186 dairy cows aged 2 to 15 years, representing ten commercial dairy herds which were chosen on a history of sporadic clinical paratuberculosis registered by the FVO. Herd sizes ranged from 5 to 35 animals. Group B included 100 dairy cows arbitrarily selected from animal patients admitted to the Clinic of Veterinary Internal Medicine, University of Zurich, Switzerland. Animals, each representing a single dairy herd, ranged in age from 2 to 11 years and were admitted to the clinic for reasons others than intestinal diseases. A history of paratuberculosis was not known. Samples were collected over a 6-months period from April until August 2002. Feces were collected from the rectum and were processed for culture within 24 h of collection. Aliquots were stored at -20 and -70°C for further investigations. Blood samples were taken from all animals. Sera were separated and stored at -20°C until use.

Microscopy

Glass-slide smears prepared from feces were stained by the Ziehl-Neelsen-technique (Ziehl, 1882; Neelsen, 1885), and examined for acid-fast staining bacteria by light microscopy under oil immersion (x1,000). Slides were read for at least 15 min for acid-fast staining bacteria (AFB).

Decontamination procedures and culture methods

Two methods of fecal decontamination were compared. Method 1 was the NaOH/oxalic acid treatment of Beerwerth (1967). Two grams of feces were homogenised with sterile sand in 50 ml of 4.0% NaOH in a mortar. Particulate matter was allowed to settle for 15 min. The supernatant was transferred into a sterile 50 ml polycarbonate screw capped tube and shaken for 10 min on a horizontal shaker (Bühler, Hechingen, Germany). After centrifugation (3,000 x g, 15 min, 20°C) the pellet was resuspended in 20 ml of 5.0% oxalic acid by thorough repeated agitation on a vortexer and was then shaken for 15 min on a horizontal shaker. The suspension was centrifuged as before, the pellet was resuspended in 4 ml of sterile saline (0.15 M NaCl) and used as inoculum. 200 µl aliquots were transferred to each of three slants of Loewenstein-Jensen (LJ) medium containing 2.0 mg/l mycobactin J and to one slant of LJ medium without mycobactin (Enclit, Oelzschau, Germany). The inoculation of LJ slants was performed as described elsewhere (Kalis et al., 1999).

Method 2 was a HPC-decontamination combined with antimicrobial agent treatment adopted from Whitlock and Rosenberger (1990) and Shin (1989). Two grams of feces were homogenised with sterile sand in 5 ml of 0.75% hexadecylpyridinium chloride (HPC, Sigma, Buchs, Switzerland) in a mortar. The mixture was transferred into a sterile 10 ml polycarbonate screw capped tube and incubated for 18 h at ambient temperature under slow agitation to assure uniform distribution (horizontal shaker, Bühler, Hechingen, Germany). The tube was centrifuged (900 x g, 30 min, 20°C) and the pellet was resuspended in 2 ml of 0.1 M phosphate buffered saline (PBS, 136.9 mM NaCl, 1.46 mM KH_2PO_4, 8.1 mM Na_2HPO_4 x $2H_2O$, 2.7 mM KCl, pH 7.4) with 100 µg/ml vancomycin, 100 µg/ml naladixic acid and 50 µg/ml amphotericin B (all purchased from Sigma). After a further 18 h incubation the suspension was used to inoculate slants as described above.

Inoculated tubes were incubated at 37°C for 16 weeks and growth was determined visually at weekly intervals. Culture tubes and samples were recorded as contaminated according to the criteria of Kalis et al. (2000). Small inocula of colonies of acid-fast staining bacteria were subcultured on LJ slants with and without mycobactin. Bacteria from pure cultures confirmed by uniform colonial and microscopic morphology were propagated in Middlebrook 7H9 broth with ADC-enrichment (Becton Dickinson, Basel, Switzerland) and containing 2.0 mg/l mycobactin J (Synbiotics Europe SAS, Munich, Germany). Harvests of pure cultures in Middlebrook 7H9 medium containing 50% glycerol (Sigma) were stored at -70°C.

Identification of *M. avium* ssp. *paratuberculosis*

MAP were presumptively diagnosed on the basis of (i) colonial morphology (small, smooth to slightly rough, opaque to whitish colonies), (ii) micromorphology (acid-fast staining, small, rod-shaped bacteria), and (iii) mycobactin dependency. Aliquots of frozen pure cultures were subjected to molecular identification by PCR-coupled RFLP analysis of the IS*1311* insertion sequence of *M. avium* (Marsh et al., 1999). The following reference strains were used: MAP strain ATCC 19698, MAP strain 6783 (bovine strain, Institute of Microbiology and Animal Infectious Diseases, Veterinary School, Hannover, Germany), and *M. avium* subspecies *avium* ATCC 25291.

PCR amplification and RFLP analysis

Genomic DNA was obtained from thawed mycobacterial stocks by incubation (56°C, 1 h) of a 50 µl aliquot in 450 µl of lysis buffer containing 100mM TRIS, 0.05% TWEEN 20 and 1 mg/ml proteinase K. Samples were then incubated at 100°C for 10 min to inactivate proteinase K. A 5 µl aliquot was used as PCR template. Two positive and negative DNA extraction controls were processed for every batch of strains. Oligonucleotide primers (M56: 5'-GCGTGAGGCTCTGTGGTGAA-3' and M119: 5'-ATGACGACCGCTTGGGAGAC-3') described by Marsh et al. (1999) were used to amplify a 608 bp fragment from the IS*1311* insertion sequence of *M. avium*. Primers were purchased from MWG Biotech (Ebersberg, Germany). Optimised reaction conditions consisted of 50 µl reaction mixtures using HotstarTaq Master Mix Kit (Qiagen, Basel, Switzerland), 0.2 µM of each primer, and 5.0 µl template DNA. All reactions were performed in a DNA thermal cycler 2400 (Perkin-Elmer, Weiterstadt,

Germany) with the following profiles: initial activation at 95°C for 15 min, followed by 37 cycles with denaturation at 94°C for 30 sec, annealing at 62°C for 15 sec, and extension at 72°C for 60 sec. The final extension step was carried out at 72°C for 7 min. PCR runs included negative and positive extraction controls. PCR products (5 µl) were separated on 1% agarose gels (Agarose NEEO, Roth, Karlsruhe, Germany) containing ethidium bromide. Amplification products were sized under UV light with reference to 100-bp DNA molecular size marker lanes (Invitrogen, Breda, Netherlands) by using a computer-aided bio-image system (BioProfil 3.1, LTF, Wasserburg, Germany). For nucleotide sequences analysis amplification products were purified with the QIAQuick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing was performed by MWG Biotech (Ebersberg, Germany).

Restriction endonucleases *Mse*I and *Hinf*I (Bioconcept, Allschwil, Switzerland) were used to cleave IS*1311*-PCR amplification products according to the manufacturer's recommendations. 20 µl aliquots of PCR products were incubated for 12 h at 37°C with ten units *Mse*I and ten units *Hinf*I. Following digestion, DNA fragments were precipitated with 3 M Na-acetate and absolute ethanol, and were resolved in 10 µl TE buffer. Restriction digests were separated electrophoretically in a 3% agarose gel (Small DNA agarose, Biozym, Oldendorf, Germany) containing 0.3 µg/ml ethidium bromide. The bio-image system was used to assess RFLP-patterns against a 100-bp DNA ladder (Invitrogen).

Serum antibody ELISA

A commercial ELISA for the detection of antibodies against the MAP-lipoarabinomannan was used (Paratuberculosis ELISA Svanovir®, Svanova Biotech, Uppsala, Sweden). The test was performed and results were interpreted according to the manufacturer's instructions with sera being tested in a 1:100-dilution in duplicate. Positive and negative control sera were included in every test. Optical densities (OD) were recorded at 450 nm by a computer-aided microplate reader (Tecan, Switzerland).

Results

Efficacy of decontamination protocols

A total of 286 individual fecal samples were decontaminated by two different methods (method 1, NaOH-oxalic acid; method 2, HPC-antibiotics), and were subsequently cultured on four slants of LJ medium each resulting in a total of 1144 cultures for each decontamination protocol. Of the 1144 method-1 cultures, 56 (4.9%) were contaminated after 4 weeks of incubation, increasing cumulatively to 100 (8.7%), 141 (12.3%), and 202 (17.6%) after 8, 12, and 16 weeks of incubation, respectively. For 9 samples (3.1%) all 4 tubes were lost due to bacterial overgrowth. By comparison, the method-2 decontamination resulted in 150 tubes (13.1%) with overgrowth of bacterial contaminants after four weeks of incubation, increasing cumulatively to 197 (17.2%), 221 (19.3%), and 246 (21.5%) after 8, 12, and 16 weeks. 21 samples (7.3%) were completely lost. The rate of contaminated culture tubes and the number of samples lost due to contamination was significantly higher in fecal samples treated with method 2 ($p<0.01$, McNemar's test). Only for one fecal sample (0.4%) all tubes from both decontamination protocols were lost due to microbial overgrowth.

Comparison of microscopic detection and culture of mycobacteria

A total of 102 individual fecal samples were arbitrarily selected from animals of group A to compare microscopic detection of AFB in fecal smears with isolation of mycobacteria in culture. AFB morphotypes resembling MAP (fairly short rods) were detected in 50 samples (49.0%). In 47 of these 50 AFB-positive smears (94.0%), only few scattered acid-fast rods occasionally in clumps were seen during the 15-min examination period. In the remaining 3 samples (6.0%), AFB (single cells or clumps) were detected in the vast majority of microscopic fields. AFB were isolated from 57 fecal samples (55.8%): 31 isolates were grown from samples with a positive microscopy for AFB, and 26 isolates were grown from microscopically negative samples. In comparison with culture, microscopy for AFB had a sensitivity and a specificity value of 54.4% and 73.0%, respectively (Table 1).

Isolation of mycobacteria

MAP was grown after 4 to 16 weeks of incubation from 15 of 186 animals (8.1%) from herds with a history of paratuberculosis (group A). Phenotypic diagnostic criteria were colonial morphology, the presence of AFB after Ziehl-Neelsen staining, and

mycobactin dependency. MAP was not isolated from feces of group-B animals. Other mycobacteria than MAP (fast-growing mycobactin independent AFB) were isolated from 86 animals of group A (46.2%) and from 48 animals of group B (48.0%) (data not shown). 10 samples contained at least two different mycobacterial strains and one sample revealed three different mycobacterial strains. A total of 145 non-MAP strains were established. All 160 mycobacterial isolates were examined by PCR amplification for the presence of the insertion element IS*1311*. A PCR amplification product of the predicted size of 608 bp was obtained from 15 isolates which had been classified as MAP phenotypically. The RFLP profile of the IS*1311*-PCR products revealed three fragments of 67, 218, and 323 bp which are characteristic for the bovine typ of MAP (Fig. 1). Nucleotide sequences of IS*1311*-PCR products were 99.9 to 100 % identical with data bank entries (Acc.-No. U16276).

Serum antibody ELISA

The test validity criteria were fulfilled in all ELISA tests performed in our study (OD pos. control >1.00, OD neg. control <10% of OD pos. control). Based on the manufacturer's interpretation criteria, animals were classified as positive, doubtful, or negative as given in Table 2. Positive ELISA reactions indicative for the presence of MAP serum antibodies were detected in 39 of 186 animals (20.9%) from dairy herds with a history of paratuberculosis (group A). MAP was grown from 6 of the 39 seropositives (15.4%). 15 sera (8.0%) revealed doubtful ELISA results. MAP was cultured from 2 animals with a doubtful serology (13.3%) and from 7 animals with a negative serology (5.3%). Positive and doubtful ELISA results were found in 9 and 8 animals, respectively from group B (9.0%, 8.0%). Culture of MAP was negative for all animals of group B. To investigate a possible influence of intestinal infections with mycobacteria others than MAP on serum reactivity with MAP lipoarabinomannan, OD values of sera from animals with a negative microscopy and a negative culture for mycobacteria other than MAP (n=26) were compared with serum OD values from animals with both, positive microscopy and culture (n=31, Table 1). The median OD values of the AFB-negative- and positive group were 0.184 and 0.160, respectively. The differences in the median values among the two groups were not significantly different (p=0.157, Mann-Whitney test).

Discussion

Cultural recovery of MAP from bovine feces requires drastic methods for the inactivation of undesirable microoganisms which could outcompete the slow-growing MAP during the subsequent long lasting culture incubation period. However, most decontamination methods designed for treating clinical samples are also detrimental to the viability of mycobacteria (Palomino and Portaels, 1998). Decontamination of bovine fecal specimens is routinely achieved by chemical treatment. A broad variety of methods employed for eliminating bacterial and fungal contaminations from bovine fecal specimens have been evaluated worldwide with most laboratories relying on the use of the NaOH/oxalic acid or the HPC decontamination (Kalis et al., 1999; Merkal et al., 1982). In comparison to NaOH/oxalic acid, HPC is suggested less detrimental to MAP (Kenefick et al., 1988; Stachelscheid, 1989). Therefore, pretreatment of bovine feces with HPC is recommended in a series of studies (Whitlock and Rosenberger, 1990; Stabel, 1997). Notwithstanding this, our comparison of the NaOH/oxalic acid method of Beerwerth (1967) with the HPC-method adopted from Whitlock and Rosenberger (1990) and Shin (1989) revealed obvious differences in adverse effects on the viability of MAP. HPC-treatment of fecal specimens resulted in a significant lower yield of MAP isolates and a significant higher rate of contaminated culture tubes than the NaOH/oxalic acid decontamination. Moreover, atypical mycobacteria which were grown from 46.8% of NaOH/oxalic acid treated specimens, were not obtained from any HPC-treated sample. Differences in the susceptibility of mycobacterial species to the detrimental effects of decontamination are well known (Portaels, 1995). Aside the studies of Kenefick et al. (1988) and Stachelscheid (1989) systematic comparisons of the HPC-decontamination and other chemical treatments with regard to the detrimental effect on MAP-viability in bovine feces have not been published. Our finding of high numbers of atypical mycobacteria in NaOH/oxalic acid treated feces lends support to the assumption that this type of decontamination is appropriate to preserve the viability of mycobacteria including MAP from bovine feces to a higher extent than HPC. Although the NaOH/oxalic acid method is laborious because of the 3-step approach in decontamination, it is applicable particularly to grow MAP from paucibacillary fecal samples thus providing a helpful tool in an eradication program for paratuberculosis (Kalis et al., 1999). Since our results of the culturability of MAP after HPC treatment of fecal specimens are in contrast to evidence from the literature, further comparative studies will be required in order to

determine the impact of different decontamination methods on the growth of MAP from bovine feces.

Advances in molecular techniques based on direct amplification e.g. of the IS*900* insertion sequence which is considered specific for MAP have allowed a rapid classification without probable biases of a culture-dependent approach (Lambrecht and Collins,1992). It has been shown very recently that mycobacteria other than MAP harbour insertion sequences which are amplifiable with several primers designed for detection of the MAP-IS*900* element (Englund et al., 2002). Since the resulting amplicons were of the same sizes as the corresponding products from MAP, the specificity of the IS*900*-PCR as a tool to identify MAP directly in clinical specimens needs to be verified. In our study, MAP were classified by using a PCR-coupled RFLP analysis of the IS*1311* insertion sequence which has the advantage to allow the differentiation of the bovine and ovine genotypic variants of MAP (Marsh et al., 1999). It would be important to test our strain collection of atypical mycobacteria for the presence of IS*900* analoga using methodologies such as those described by others (Cousins et al., 1999; Englund et al., 2002).

In paratuberculosis control programs, culture of MAP and serodiagnosis should be performed concurrently. Since the strength of the antibody response depends directly on the disease progress, results of fecal culture and serology are not necessarily complementary. Sensitivity of serology, i.e. ELISA is high in clinically affected animals (Collins and Sockett, 1993; Sweeney et al., 1995; Whitlock et al., 2000) but 63 to 91% of asymptomatic cattle with a culture-confirmed paucibacillary fecal shedding of MAP remain unidentified as infected by serology (Eamens et al., 2000; Muskens et al., 2003). This was also observed in our study: four animals with clinical paratuberculosis revealed a strong positive ELISA reactivity. Out of a total of 15 MAP-culture positive cows, only six animals (40.0%) exhibited a positive antibody response, thus corroborating the low sensitivity of serology in subclinically infected cows. By repeated serological testing over nine months, seroconversion has been observed in approximately 64% of formerly seronegative MAP-shedding cattle (Eamens et al., 2000). Thus, the lack of reactivity in sera retrieved from MAP-shedders represents animals prior to the development of a significant humoral immune response. Out of the 186 cows from herds with a history of paratuberculosis, 39 (20.9%) were seropositive and this percentage was significantly higher than in the group of arbitrarilary selected 100 cows representing 100 dairy herds without any

history of paratuberculosis mainly from the eastern part of Switzerland (9.0%). Out of 39 seropositives from affected herds, only six cows (15.4%) tested culture-positive. This is also reflected by the study of Muskens et al. (2003) who found 17.3% of 371 seropositive cows culture-positive. The positive and negative predictive values of the ELISA used in our study are high (Jark et al., 1997). Therefore it seems reasonable to consider our serologic results as true, and we agree with other authors who stated that only a low percentage of ELISA-positive cattle can be confirmed as MAP shedders by fecal culture mainly due to intermittent shedding of varying numbers of MAP (Muskens et al., 2003). Nevertheless, it is tempting to assume that false-positive ELISA results may in part cause the divergence between serology and culture especially in view of the high percentage of animals with a fecal shedding of mycobacteria designated as "atypical". Infection of cattle with atypical mycobacteria, e.g. *M. kansasii* can evoke an immune response with highly cross-reactive mycobacterial antibodies (Kazda, 1969; Schliesser, 1965). Antibodies against non-MAP are known as a cause of false-positive reactions in MAP serology but can be avoided by absorption of sera with *M. phlei* (Yokomizo et al., 1985). The ELISA test used in our study was performed with unabsorbed sera as recommended by the manufacturer. Interestingly, the presence of atypical mycobacteria in feces as confirmed by microscopy and culture was not correlated with any increased ELISA reactivity in these animals. These results indirectly provide evidence for the validity of positive ELISA reactivities observed in animals without a MAP-positive fecal culture.

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Tables and Figure

Table 1. Comparison of microscopy and culture for the detection of acid-fast staining bacteria in bovine fecal samples

AFB[1]-microscopy	Culture of mycobacteria		total
	positive	negative	
positive	31	19	50
negative	26	26	52
total	57	45	102

[1] acid-fast bacteria

Table 2. Comparison of culture and serology

	ELISA results group A[1] (n = 186)			ELISA results group B[2] (n = 100)		
Culture results	positive	doubtful	negative	positive	doubtful	negative
MAP[3]	3.2[5]	1.1	3.8	0	0	0
non-MAP[4]	9.1	2.7	33.9	1.0	5.0	42.0
no mycobacteria	8.6	4.3	33.3	8.0	3.0	41.0
total	20.9	8.1	71.0	9.0	8.0	83.0

[1] samples from herds with cases of clinical paratuberculosis

[2] samples from herds without a history of paratuberculosis

[3] *M. avium* ssp. *paratuberculosis*

[4] mycobacteria other than MAP

[5] results in %

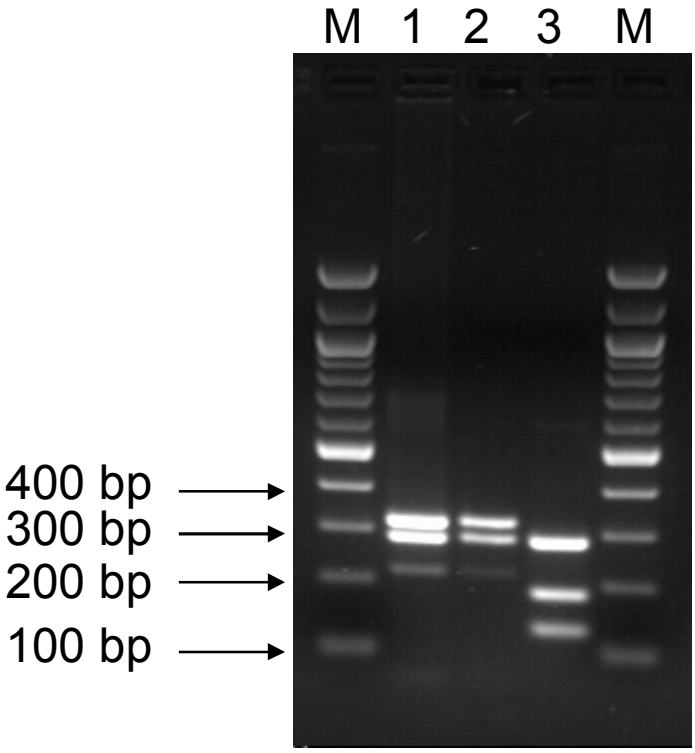

Figure 1. *Mse*I/*Hinf*I restriction endonuclease analysis of the IS*1311* insertion element

Lane M, DNA size marker (100-bp DNA ladder)
Lane 1, *M. avium* ssp. *paratuberculosis* ATCC 19698
Lane 2, MAP-isolate
Lane 3, *M. avium* ssp. *avium*

2. Danksagung

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